B4

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 348 173 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 89306263.8

(22) Date of filing: 21.06.89

(51) Int. Cl.⁴: G 01 N 33/53
G 01 N 33/542, G 01 N 33/535

(30) Priority: 22.06.88 US 209916

(43) Date of publication of application:
27.12.89 Bulletin 89/52

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: MICROGENICS CORPORATION
2380A Bisso Lane
Concord California 94520 (US)

(72) Inventor: Khanna, Pyare L.
864 Gregory Court
Fremont California 94359 (US)

Friedman, Stephen B.
107 Sturbridge Lane
Chapel Hill North Carolina 27516 (US)

Kates, David S.
816 San Tomas Street
Davis California 95616 (US)

(74) Representative: Harrison, David Christopher et al
MEWBURN ELLIS 2 Cursitor Street
London EC4A 1BQ (GB)

(54) Liquid single reagent assay.

(57) Normally interacting reagents are both present in a liquid single reagent but complex formation is inhibited using a surfactant; the inhibition is reversed in the presence of analyte by adding a cyclodextrin. The invention finds particular use in diagnostic immunoassays.

EP 0 348 173 A2

. Bundesdruckerei Berlin

Description

LIQUID SINGLE REAGENT ASSAY

The present invention relates to immunoassay reagents, and, in particular, to reagents for competitive inhibition assays.

A wide variety of immunoassays have been based on competitive inhibition where analyte in the sample competes for a fixed amount of anti-analyte antibody with a known amount of analyte, usually labeled analyte, present in the assay medium. Enzyme labels are often used in competitive inhibition assays, where binding of anti-analyte antibody with an enzyme-analyte conjugate changes, usually decreases, the rate of enzyme-catalyzed breakdown of substrate.

A number of such assays have been based on the ability of fragments of β-galactosidase to complement and form active enzyme. In particular, a β-galactosidase enzyme donor (ED) combines with a β-galactosidase enzyme acceptor (EA) to form active β-galactosidase enzyme. Conjugating a small analyte or an analyte analogue to the ED at certain sites does not affect the rate of β-galactosidase catalyzed activity. However, when the ED-analyte conjugate is bound by anti-analyte antibody, the enzyme-catalyzed reaction rate during the initial phase of the reaction is reduced. This reduction in enzyme-catalyzed reaction rate has been used to quantitate the determination of a plurality of analytes where ED-analyte conjugate present in a assay medium and analyte present in the sample compete for anti-analyte antibody prior to the addition of EA. The β-galactosidase-catalyzed reaction rate increases as the amount of analyte present in the sample increases.

With competitive assays, the labeled analyte and anti-analyte antibody generally cannot be mixed prior to addition of the sample. In complementation assays, usually the ED and EA are separated until after incubation of ED-analyte conjugate and sample with anti-analyte antibody. This necessitates careful delivery by the end user of any reagent in the assay used in limiting quantity. Further, such assays are not readily adaptable to automation since most analyzers dispense only a single analyte-specific reagent and a common reagent to each sample well.

Relevant Literature

Modified β-galactosidase enzyme donors and enzyme acceptors have been prepared by chemical synthesis and recombinant engineering. The modified fragments retain β-galactosidase activity upon complementation and facilitate production of and attachment of analyte to the fragments. See for example U.S. Patent No. 4,708,929 and the articles cited therein.

Cyclodextrins are commercially available and are well known compounds which form inclusion compounds (clathrates) which are capable of trapping a number of organic molecules. For a description of their properties and methods of production, see, for example, Bender et al., Cyclodextrin Chemistry, Springer-Verlag, New York, 1978 (a 96-page book); French, Adv. Carbohydr. Chem. (1957) 12:189-260; Thoma et al., Starch: Chemistry and Technology, Vol. 1, Whistler et al., Eds., Academic Press, New York, 1965, pp. 209-249; and Cramer et al., Naturwiss. (1967) 154:625-635. The compounds are naturally occurring and are obtained from the action of Bacillus macerans amylase on starch.

EPO application No. 87302511.8 to Gibbons et al. Publication No. 0 243 001 describes a single reagent assay method wherein a specific binding pair (sbp) member is reversibly confined in a material that temporarily renders the confined sbp member incapable of binding with its complementary member. In a preferred embodiment, an enzyme-hapten conjugate is confined in a liposome and the assay reaction is initiated by the addition of a surfactant.

SUMMARY OF THE INVENTION

A method is provided for combining normally interacting reagents in a liquid single reagent and preventing complex formation using a surfactant and then reversing the inhibition by adding a cyclodextrin. The method finds particular use in diagnostic immunoassays. Reagents facilitating the invention are also provided.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A method is provided wherein reaction of complementary members of a specific binding pair to form a specific binding pair complex is reversibly inhibited by use of a surfactant. Reaction is then initiated by adding a cyclodextrin. The method can be used any time it is desirable that a mixture of the complementary members of a specific binding pair are combined prior to initiation of complex formation. The method finds particular application in diagnostic assays.

The method comprises providing, in a process which uses complex formation between complementary members of a specific binding pair, a prepared liquid single reagent comprising a reaction-inhibiting amount of a surfactant and the complementary members of the specific binding pair. In performing the process, a sufficient amount of a cyclodextrin is added to the single reagent to allow complex formation.

The liquid single reagent may be employed whenever combining the complementary members of a specific

binding pair without initiating complex formation is desirable, since the inhibition is reversible. This reversible inhibition of complex formation will be desirable whenever accurate measurement of the complementary members of a specific binding pair is required, such as where one or both members are present in limiting quantity. The method finds application in diagnostic assays where one or both complementary members of a specific binding pair are combined with the sample in a assay medium as a first step of an assay procedure. In accordance with the subject invention, the components which are to be mixed with the sample can be provided as a liquid single reagent where the complementary members of a specific binding pair are combined without initiating complex formation. Other than eliminating measurement steps and adding the cyclodextrin solution to initiate complex formation, the assay methods, conditions and reagents using the single reagent formulation will not differ significantly from those of the prior art.

As stated previously, the liquid single reagent comprises a reaction-inhibiting amount of a surfactant and the complementary members of a specific binding pair. Other components of the assay medium, e.g. enzyme substrate, buffer solution, etc. may also be included in the liquid single reagent composition. These additional components may vary depending on the particular assay protocol. Stabilizers, bactericides or preservatives which do not interfere with the function of the assay components may also be present. While the liquid single reagent will be used in liquid form in an assay, the liquid single reagent may be provided as a liquid or dried or lyophilized to form a powder. The powder composition can be formulated to be reconstituted in buffer, water or the sample, depending on the assay.

The liquid single reagent may be used in an assay method to quantitate any analyte determined by prior art immunoassay or complementation methods including proteins, drugs, polynucleotides, or polysaccharides. The method can be used with any aqueous sample containing the analyte. The analyte may be a small analyte, a hapten, present in a bodily fluid, usually in patient serum, plasma, saliva, urine, or whole blood, or a larger analyte such as proteins. Sample pretreatment will follow conventional procedures.

Numerous specific binding pairs which may find use in diagnostic assays are known. Usually, at least one of the complementary members of the specific binding pair will be a protein or protein fragment, e.g. enzyme donor/enzyme acceptor, antigen/antibody, lectin/sugar, etc. many competitive inhibitition assays use a known amount of an analyte reagent, usually a labeled analyte, that competes with analyte present in the sample for a predetermined amount of the reciprocal binding pair member. As the receptor, an antibody will usually be employed, either polyclonal or monoclonal. Alternatively, a receptor which specifically binds an analyte can be used in place of antibody, e.g. vitamin B12-intrinsic factor, thyroxin-thyroxine binding globulin, and folic acid-folate binding protein. Either member of the specific binding pair may be bound to a label. The label may be an enzyme, fluoroscer, dye, substrate, or chemiluminescer. Desirably the label is an enzyme bound to the analyte or an analyte analogue to produce an enzyme-analyte conjugate.

There are a number of surfactants which may be used to prevent the reactions of complementary members of a specific binding pair and which can be neutralized by a cyclodextrin. For example, a number of biological detergents (surfactants) are listed as such by Sigma Chemical Company on pages 316-321 of its 1988 Catalog of Biochemical and Organic Compounds. Such surfactants are divided into four basic types: anionic, cationic, zwitterionic, and nonionic. Examples of anionic detergents include alginic acid, caprylic acid, cholic acid, 1-decanesulfonic acid, deoxycholic acid, 1-dodecanesulfonic acid, N-lauroylsarcosine, and taurocholic acid. Cationic detergents include dodecyltrimethylammonium bromide, benzalkonium chloride, benzyldimethylhexadecyl ammonium chloride, cetylpyridinium chloride, methylbenzethonium chloride, and 4-picoline dodecyl sulfate. Examples of zwitterionic detergents include 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (commonly abbreviated CHAPS), 3-[(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (generally abbreviated CHAPSO), N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and lyso-α-phosphatidylcholine. Examples of nonionic detergents include decanoyl-N-methylglucamide, diethylene glycol monopentyl ether, n-dodecyl β-D-glucopyranoside, polyoxyethylene ethers of fatty acids (particularly C_{12}-C_{20} fatty acids, (e.g., sold under the trade name Triton), ethylene oxide condensates of fatty alcohols e.g. sold under the name Lubrol), polyoxyethylene sorbitan fatty acid ethers (e.g., sold under the trade name Tween), and sorbitan fatty acid ethers (e.g., sold under the trade name Span).

The surfactant will be present in the liquid single reagent in a concentration at least sufficient to inhibit complex formation between the complementary members of a specific binding pair. Desirably, the concentration will be insufficient to denature the specific binding pair members, the sample analyte or any other assay reagents. In any case the surfactant effects will be reversed by use of a cyclodextrin and the assay medium components will not be permanently denatured. A preferred surfactant is a nonionic detergent, desirably polyoxyethylene esters of fatty acids, particularly C_{12}-C_{20} fatty acids, (e.g., sold under the trade name Triton), which is used at about 0.2% to about 1.0% (w/w), usually about 0.2% to about 0.5% (w/w), of the liquid single reagent formulation.

Cyclodextrins are cyclic anyloses. Three types are known: a cyclohexaamylose (α-cyclodextrin), a cycloheptaamylose (β-cyclodextrin), and a cyclooctaamylose (γ-cyclodextrin). Although all three of the cyclodextrins are effective in removing surfactants, advantages are achieved by matching the size of the portion of the surfactant complexed in the available interior space of the cyclodextrin. Large surfactants defining a relatively large molecular volume, such as those derived from cholic acid, are therefore most readily removed using γ-cyclodextrin, which has the largest interior space. Smaller surfactants, such as fatty acid salts and fatty sulfonates, which are elongated chains having a relatively small molecular cross-section, are most readily removed with α-cyclodextrin, which has the smallest interior space of the three cyclodextrins.

The use of a sufficient amount of a cyclodextrin reverses surfactant-induced inhibition of complex formation, thus neutralizing the surfactant, and allows complex formation between the specific binding pair members. It is preferred to use a molar excess of the cyclodextrin in relation to detergent in order to substantially completely sequester the surfactant and reverse the effect of the detergent. The cyclodextrin will be added in a volume of from about 0.1 to 5, usually 0.5 to 3 times the volume of from the sample and liquid single reagent. Conveniently the cyclodextrin is prepared as an aqueous solution, referred to as a "start" solution, in a concentration of from about 1.2 to 10 times the concentration desired in the assay medium, generally about 1.5 to 5.0 times the desired final concentration. The start solution can be prepared in any buffer suitable for use in an assay or suitable when mixed with the solution containing the other components of the assay medium. Generally satisfactory start solutions can be prepared as aqueous solutions containing from about 0.5% to about 5.0%, usually about 1.0 to about 2.0% (w/v) cyclodextrin.

While use of a cyclodextrin is preferred to reverse the surfactant-induced inhibition of complex formation, other surfactant-inhibition agents also find use. For example, the surfactant inhibition can be reversed by the addition of an excess of protein. Alternatively, some surfactants are known to neutralize the effects of other surfactants. See, for example, Dimitriadis, Anal. Bioch. (1979) 98:445-451.

The liquid single reagent composition will vary widely, depending upon the nature of the reagents and the desired ratio of the complementary specific binding pair members. Also, the amount of other materials may affect the concentration of the sbp members and vice versa. Each of the complementary members of the specific binding pair will generally be present in $5x10^{-6}$ to 0.1% by weight of dry ingredients (excluding fillers and excipients). In the case of enzyme labels, if substrate is present, it will be present in an amount of about 1 to 15%. Buffer will usually comprise 5 to 30%. Finally, the surfactant will be present in an amount of from about 10 to 30%.

A preferred embodiment for determining the amount of analyte present in a sample comprises combining a liquid single reagent comprising an enzyme-analyte conjugate, an anti-analyte antibody, and a reaction-inhibiting amount of a surfactant, with the sample, enzyme substrate and a sufficient amount of a cyclodextrin to initiate complex formation. The enzyme-catalyzed reaction rate during the assay measurement differs, usually decreases, when the enzyme conjugate is bound by antibody, e.g. glucose-6-phosphate dehydrogenase (G6PDH) or β-galactosidase present as enzyme donor (ED) and enzyme acceptor (EA) fragments. The cyclodextrin is not added to the single reagent components prior to addition of the sample. The rate of enzyme-catalyzed reaction is determined as indicative of the amount of analyte present in the sample. Assays which utilize G6PD-analyte conjugates are described in U.S. Patent No. 3,817,837. Assays which utilize β-galactosidase ED-analyte conjugates are described hereinafter and in U.S. Patent No. 4,708,929.

The β-galactosidase complementation assay method utilizes a liquid single reagent comprising in a reaction buffer, a β-galactosidase ED-analyte conjugate, an anti-analyte antibody and a surfactant in a concentration sufficient to inhibit complex formation between analyte and anti-analyte antibody and complementation between ED and EA. The single reagent may additionally contain a β-galactosidase EA and/or enzyme substrate. Alternatively, those components which are not specific for a particular analyte can be provided as a second, common reagent. In the case of physiological fluids, other than the removal of particulates, no pretreatment of the sample will usually be performed for purposes of the instant assay method.

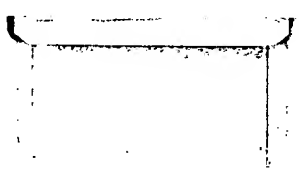
The method comprises combining a liquid single reagent comprising a β-galactosidase ED-analyte conjugate, an anti-analyte antibody, a reaction-inhibiting amount of a surfactant, and optimally as part of the single reagent or separately, a β-galactosidase EA and enzyme substrate with the sample and a cyclodextrin. The cyclodextrin is added to the liquid single reagent in the presence of sample, either together with or following addition of sample. The β-galactosidase-catalyzed substrate transformation to product is determined in a preselected time period as indicative of the amount of analyte present in the sample.

The enzyme donor and enzyme acceptor are partial sequences of β-galactosidase. Either partial sequence may be mutated to facilitate preparation of the sequence, attachment of an analyte or the like. The enzyme acceptor and enzyme donor analyte conjugate are characterized by forming an active enzyme complex when brought together. When the enzyme donor conjugate is bound to anti-analyte antibody the observed enzyme activity is different from that observed in the absence of anti-analyte antibody. Thus, the availability of antibody to bind with enzyme donor conjugate will vary with the amount of analyte in the medium.

β-Galactosidase enzyme donors and acceptors are described in U.S. Patent No. 4,708,929, which disclosure is incorporated herein by reference, as are analytes and enzyme donor-analyte conjugates. Copending U.S. Patent Application, Serial No. 151,412 filed February 2, 1988, describes reaction conditions and reagents for complementation assays. The conditions of the assay described in that application are applicable to the subject invention.

The assay conditions and the reaction buffer used provide for complementation between enzyme donor and enzyme acceptor. In general, physiological buffers such as phosphate buffered saline, tris buffer and like buffers are useful. The ionic strength is not critical. A preferred buffer comprises about 100 mM to about 300 mM $NaPO_4$, about 5 mM to about 10 mM EGTA, and about 10 mM to 20 mM NaN_3 having a pH of between 6 and 8. The temperature for the assay will usually be at least about 20°C, preferably elevated, but below 60°C. Enzyme assays are generally conducted at between room temperature (25°C) to less than about 40°C, most usually about 37°C. The assays are performed at atmospheric pressure.

The concentration of enzyme donor conjugates in the assay medium will usually be in the range of about 1 nM to about 60 nM, more usually about 5 nM to about 50 nM, most usually 10 to 25 nm. The enzyme acceptor



will usually be in substantial molar excess. The EA concentration will vary from about 0.5 to about 5.0 μM, usually about 1 to about 2 μM. The molar ratios of enzyme donor conjugate to enzyme acceptor will usually be 1:30 to 1:80, more usually 1:50 to 1:60. The concentration of the enzyme donor-analyte conjugate will usually exceed the highest concentration of the analyte anticipated to be encountered in the sample.

The optimal ratio of ED-analyte conjugate and anti-analyte antibody will be determined in the presence of EA so as to span the range of assay ligand and also to minimize the background activity. The response of the enzyme-catalyzed reaction rate to analyte concentration in relation to background level is optimized.

The ratio of the concentration of ED-analyte conjugate and anti-analyte antibody will be such as to substantially achieve minimum enzyme rate under assay conditions, while maintaining linearity of the rate varying with analyte concentration over the desired assay range. Usually the concentrations of antibody and conjugate will be within at least 85%, more usually within at least 95% of the concentration necessary to optimize conditions.

Varying amounts of sample can be used. When the sample is serum, usually the sample will comprise from about 1% to about 10% of the volume of the assay medium, more usually about 2% to about 5%.

An enzyme substrate is employed that when cleaved by the enzyme results in a change in the amount of light absorbance (optical density) or emission of the assay medium. That is, cleavage of the substrate results in the appearance or disappearance of a colored or fluorescent product. Preferred enzyme substrates include ortho-nitrophenyl galactoside (ONPG) and chlorophenylred-β-galactoside (CPRG). ONPG and CPRG absorbance values are measured at 420 nm and 574 nm, respectively. ONPG, CPRG and other comparable enzyme substrates are commercially available. ONPG will generally be used at a concentration of from about 0.5 to about 2.0 mg/ml, more usually about 1.0 to about 1.5 mg/ml. Using ONPG or CPRG, the assay medium will conveniently be incubated for from about 1 to 10 min., usually 1 to 4 min., most usually 1 to 3 min. following addition of the cyclodextrin to a first determination of optical density. There will usually be 1 to 10, more usually 3 to 8, most usually 3 to 6 min., between the first and second reading.

To perform the assay, a liquid single reagent is prepared. The sample may be combined with the single reagent to form an assay medium and incubated at about 37°C for from about 10 to about 30 min. prior to addition of the cyclodextrin or the cyclodextrin may be added at the same time as the sample. Following addition of the cyclodextrin start solution and any assay components not present in the liquid single reagent, the assay medium is incubated for a predetermined period of time. The difference in the optical density of the assay medium at two preselected time points is determined as indicative of the amount of analyte in the sample. The amount of enzyme activity in the medium is determined as indicative of the amount of analyte present in the sample in accordance with standard techniques for measuring β-galactosidase activity.

A convenient way of calibrating the assay is to prepare a graph of the rate of optical density change versus concentration using control samples having known analyte concentrations. The analyte control samples are conveniently prepared solutions having 0% analyte and having an infinite concentration of analyte, i.e. an analyte concentration such that further addition of analyte does not affect the reaction rate. Desirably, one or more analyte controls of intermediate concentration, conveniently in the normal range of the sample, will also be used to prepare a standard curve.

A kit containing reagents facilitating the present invention is also contemplated. The kit comprises the complementary members of a specific binding pair and a surfactant in a first container, where usually the specific binding pair is analyte/anti-analyte antibody. A cyclodextrin is provided in a second container. For use in a complementation assay, the first container comprises ED-analyte conjugate, anti-analyte antibody, and a surfactant. The first container may additionally contain other reagents present in the assay mixture such as enzyme substrate and EA. Conveniently however, the common reagents useful for a plurality of analytes are present in a third container or with the cyclodextrin. The kit may additionally comprise one or more analyte controls at varying concentrations in the anticipated sample concentration range.

The reagents can be formulated in liquid form or in dry form as a powder or a unit dosage tablet containing a quantity of reagents suitable for assay of one sample. The dry reagent(s) may contain buffer salts or buffer can be provided for reconstituting the dry reagent. The dry reagent may be formulated with conventional additives such as stabilizers, drying agents, excipients and the like.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Reagents Example 1 - T4 Assay

Reagents

T4 Reconstitution Buffers

ED Buffer

60 mM PO_4^{3-}	Buffer
19.4 mM	NaN_3
10 mM	EGTA
0.416 mM	ANS
	pH = 7.0

EA Buffer

90 mM PO_4^{3-}	Buffer
19.4 mM	NaN_3
4.8 mM	$Mg(OAc)_2 \cdot 4H_2O$
10 mM	EGTA
	pH = 7.6
Triton X-301	As detergent
ONPG	As enzyme substrate
EA22	Described in U.S. Patent No. 4,708,929
ED_4-T_4	Prepared from Thyroxine derivative and ED_4, as described in U.S. Patent No. 4,708,929
Monoclonal anti-T_4 antibodies	
β-Cyclodextrin	Available commercially from Sigma Chemical Company
EGTA	Ethylene-bis-oxy-ethylenenitrilo tetraacetic acid

Assay Procedure

A 5 ml reagent solution was prepared by mixing the following components.
3323 µl of ED buffer
312.5 µl of a 4% Triton X-301 solution
(dilution of 20% reagent stock with ED buffer to obtain 4% solution)
750 µl of 10 mg/ml stock solution of ONPG in ED buffer
102.4 µl of 6 X 10^{-5} M EA22
200 µl of a 1:300 dilution of 1.35 X 10^{-4} M ED_4-T_4 in ED buffer
312.5 µl of a 1:100 dilution of anti-T_4
Antibody in ED buffer
A 1% β-cyclodextrin "start" solution was made using 0.50 g in 50 ml of EA buffer. 10µl of a serum calibrator ranging in concentration from 0, 2.5, 5, 10, 20 24, 200 µg/dl was added to 100 µl of the reagent solution and incubated 15 min. at 37°C (oven incubator). The reaction was initiated in a Baker Encore Chemistry Analyzer by addition of 200 µl of β-cyclodextrin start solution. Absorbance was read at 420 nm at 360 and 540 sec. following addition of cyclodextrin.

Results

The results are illustrated in Table 1. As used in the table, the percentage of modulation is the difference between any two calibrators; i.e., the net difference divided by the rate of the higher calibrator.

Table 1

T4 (µg/dl)	Δ540-360 sec (mA)	% Modulation
0	64	0
2.5	72	11.11
5	80	20.00
10	96	33.33
20	118	45.76
24	119	46.22
200	178	64.04
∞	211	69.76

As shown in Table 1, the assay is useful between concentrations of 0 and 20 µg/dl of T4 in the patient sample.

Example 2 - B12 Assay

Reagents

B12 Assay Liquid Single Reagent Solution
ED_4-B_{12} Conjugate - 3 nM
Intrinsic Factor - 1:6000 dilution
Substrate (CPRG) - 1.3 mg/ml
Triton - 0.2%, 0.4% or 0.8% in ED buffer as described in Example 1

Common Reagent Solution
EA_{22} - 1.0×10^{-6}M
β-cyclodextrin - 1.0% in EA buffer as described in Example 1

Assay Procedure

10 µl sample (0 or 1 µg/ml analyte control sample) was mixed with 100 µl of single reagent solution and 150 µl of common reagent solution. The absorbance change was read at 60 and 180 seconds following addition of cyclodextrin.

Results

The results are shown in Table 2. As used in the table, the net rate is defined as the difference in zero and high calibrator rate, and percentage inhibition is defined as the ratio of net rate to the high calibrator rate.

Table 2

In the Absence of Cyclodextrin

Triton Conc.	Rate 1-3 Min. Zero Calib.	High Calib.	Net. Rate	% Inhibition
0%	42	161	119	73.9%
0.2%	-25	-21	N/A	N/A
0.4%	-55	-54	N/A	N/A

In the Presence of Cyclodextrin

Triton Conc.	Rate 1-3 Min. Zero Calib.	High Calib.	Net. Rate	% Inhibition
0%	11	79	68	86.1%
0.2%	35	137	102	74.5%
0.4%	28	112	84	75.0%

The results indicate that Triton X-301 concentrations greater than about 0.2% effectively inhibited complementation.

Example 3 - Theophylline Assay

Reagents

Theophylline Liquid Single Reagent Solution
Prepared in 0%. 0.2%, or 0.3% Triton X-301:
[ED_4-theophylline conjugate] = 46 nm
[Anti-theophylline antibody] = 1:900 dilution
Substrate CPRG (chlorophenol red galactoside) = 0.5 mg/ml

Common Reagent Solution
(EA_{22}) = 1.2 µM containing 0, 0.5, 1 or 2% β-cyclodextrin (β-cd)

Sample
zero or 200 µg/ml of analyte control

Assay Procedure
10 µl sample was mixed with 200 µl theophylline single reagent solution along with 150 µl of common reagent. The change in absorbance was read at 4 and 8 minutes.

Results
The results are shown in Table 3 which illustrate the values for 0 and 200 µg/ml (infinite dose) calibrators with 0, 0.2 and 0.3% Triton surfactant. The percentage of inhibition of the reaction rate was calculated as follows. The change in optical density of a 0% analyte control sample was subtracted from the change in optical density of a 100% analyte control sample. That number was divided by the change in optical density for a 100% analyte control to determine the percentage of inhibition. Each Triton concentration has two charts. One chart is for rates at 0 dose in the presence of varying cyclodextrin concentrations [CD]; the other is for an infinite dose.

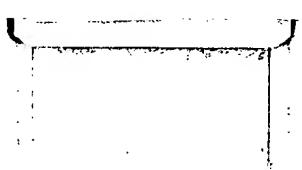


Table 3

0% Triton X-301 zero dose

[β-cd]	240 sec	480 sec	Δ480-240	% Inhibition
0%	267	975	708	0.47
0.50%	205	754	549	0.51
1%	158	566	408	0.52
2%	98	303	205	0.59

0% Triton X-301 200 dose

[β-cd]	240 sec	480 sec	Δ480-240
0%	264	1799	1335
0.50%	388	1507	1119
1%	299	1149	850
2%	193	695	502

0.2% Triton X-301 zero dose

[β-cd]	240 sec	480 sec	Δ480-240	% Inhibition
0%	68	93	25	0.34
0.50%	216	682	466	0.52
1%	196	677	481	0.53
2%	156	474	318	0.55

0.2% Triton X-301 200 dose

[β-cd]	240 sec	480 sec	Δ480-240
0%	102	140	38
0.50%	421	1385	964
1%	384	1416	1032
2%	290	991	701

0.3% Triton X-301 zero dose

[β-cd]	240 sec	480 sec	Δ480-240	% Inhibition
0%	72	79	7	0.56
0.50%	169	332	163	0.55
1%	222	734	512	0.55
2%	181	540	359	0.56

0.3% Triton X-301 200 dose

[β-cd]	240 sec	480 sec	Δ480-240
0%	99	115	16.
0.50%	333	697	364
1%	443	1569	1126
2%	327	1137	810

As shown in the table, 0.2% to 0.3% Triton X-301 was effectively neutralized by 1% to 2% β-cyclodextrin.

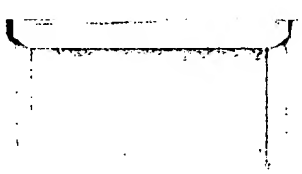
As shown by the foregoing examples, the present assay method is fast and accurate, and requires minimal manipulations and a shortened incubation period. The formulation of the reagent solution is particularly convenient, as the user need only mix a few reagents with the sample to initiate the assay. The liquid single reagent mixture is also suitable for use in autoanalyzers.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto.

Claims

1. A liquid single-reagent composition for assaying in analyte, wherein said analyte is a member of a specific binding pair comprising said analyte and a complementary binding pair member to said analyte, comprising:
   (a) said complementary member of said specific binding pair;
   (b) an enzyme donor/analyte conjugate; and
   (c) a surfactant in an amount sufficient to inhibit binding between said conjugate and said complementary member of said specific binding pair.
2. The reagent composition of Claim 1, wherein said enzyme donor/analyte conjugate is a β-galactosidase donor/analyte conjugate.
3. The reagent composition of Claim 1, wherein said specific binding pair is an antigen/antibody pair.
4. The reagent composition of Claim 1, wherein said surfactant is a non-ionic detergent.
5. The reagent composition of Claim 1, wherein said reagent further comprises an enzyme acceptor and, optionally, an enzyme substrate for the enzyme performed by the complementation reaction between said enzyme acceptor and said enzyme donor.
6. A kit for use in an amino assay, comprising:
   (a) in a first container, the liquid single reagent composition of Claim 1; and
   (b) in a second container a cyclodextrin.
7. The kit of Claim 6, wherein said first container additionally comprises an enzyme acceptor and, optionally, an enzyme substrate for the enzyme formed by complementation of said enzyme donor and said enzyme acceptor.



8. A method for determining the amount of analyte present in a sample, wherein said analyte is a member of a specific binding pair comprising said analyte and a complementary binding pair member for said analyte, said method comprising:

(a) forming an assay medium by combining:

(1) the reagent composition of Claim 1;

(2) a cyclodextrin in an amount sufficient to reverse the effects of said surfactant on binding between said conjugate and said complementary member of said specific binding pair;

(3) said sample;

(4) an enzyme acceptor; and

(5) an enzyme substrate for the enzyme formed by complementation of said enzyme donor and said enzyme acceptor;

with the proviso that said cyclodextrin is not added to said assay medium prior to addition of said sample; and

(b) determining rate of reaction of said substrate with said enzyme as indicative of the amount of analyte present in said sample.

9. The method of Claim 8, wherein said reagent composition further comprises said enzyme acceptor and, optionally, said enzyme substrate in a single liquid reagent prior to combining with other members of said assay medium.

10. The method of Claim 9, wherein said specific binding pair is an antigen/antibody pair.

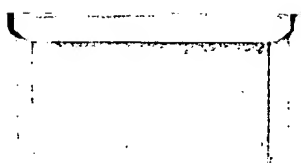
11. The method of Claim 9, wherein said rate is determined by measuring a change in light absorbance or emission as affected by a product of the enzyme-catalyzed reaction.

12. The method of Claim 9, wherein said enzyme is β-galactosidase.

13. The method of Claim 9 is a surfactant is a non-ionic detergent and said cyclodextrin is β-cyclodextrin.

14. The method of Claim 9, wherein said sample is serum and said serum comprises from about 1 to about 10% by volume of said assay medium.

15. The method of Claim 1, wherein said analyte is B12, theophylline, or T4.



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 348 173**
A3

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: **89306263.8**

(22) Date of filing: **21.06.89**

(51) Int. Cl.[5]: **G01N 33/53, G01N 33/542, G01N 33/535**

(30) Priority: **22.06.88 US 209916**

(43) Date of publication of application:
27.12.89 Bulletin 89/52

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(88) Date of deferred publication of the search report:
24.10.90 Bulletin 90/43

(71) Applicant: **MICROGENICS CORPORATION**
2380A Bisso Lane
Concord California 94520(US)

(72) Inventor: **Khanna, Pyare L.**
864 Gregory Court
Fremont California 94359(US)
Inventor: **Friedman, Stephen B.**
107 Sturbridge Lane
Chapel Hill North Carolina 27516(US)
Inventor: **Kates, David S.**
522 Oeste Drive
Davis California 95616(US)

(74) Representative: **Harrison, David Christopher et al**
MEWBURN ELLIS 2 Cursitor Street
London EC4A 1BQ(GB)

(54) **Liquid single reagent assay.**

(57) Normally interacting reagents are both present in a liquid single reagent but complex formation is inhibited using a surfactant; the inhibition is reversed in the presence of analyte by adding a cyclodextrin. The invention finds particular use in diagnostic immunoassays.

EP 0 348 173 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 30 6263

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	EP-A-0 286 367 (MICROGENICS CORP.) * The entire document *	1-15	G 01 N 33/53 G 01 N 33/542 G 01 N 33/535
A,D	EP-A-0 243 001 (SYNTEX (U.S.A.) INC.) * The entire document *	1-15	
A	THE JOURNAL OF THE CHEMICAL SOCIETY, FARADAY TRANSACTIONS 1, vol. 82, no. 5, May 1986, pages 1271-1287, London, GB; A. HERSEY et al.: "Mechanism of inclusion-compound formation for binding of organic dyes, ions and surfactants to alpha-cyclodextrin studied by kinetic methods based on competition experiments" * The entire document *	1,4,8	
A	THE JOURNAL OF NEUROCHEMISTRY, vol. 26, no. 1, January 1976, pages 217-219, New York, NY, US; J.W. CALLAHAN et al.: "Effects of detergents on the hydrolysis of glycolipids by beta-galactosidase" * The entire document *	1,4,8	TECHNICAL FIELDS SEARCHED (Int. Cl.4) G 01 N
A	EP-A-0 055 869 (H.A. THOMA) * The entire document *	8-15	
A	CLINICAL CHEMISTRY, vol. 32, no. 9, September 1986, pages 1637-1641, Washington, DC, US; D.R. HENDERSON et al.: "CEDIA TM, a new homogeneous immunossay system"		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-08-1990	DOEPFER K-P.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)